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Investigation of CerbB2 at mRNA level in patients with gastric adenocarcinoma

The mRNA-level research of CerbB2 in gastric adenocarcinoma

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Abstract
Aim: Gastric adenocarcinomas take place near the top regarding mortality due to cancer. This study aims to validate IHC results with the RT-PCR method and to evaluate their contribution to confirm the absolute score of CerbB2 situation in tissues.
Material and Methods: We analyzed 80 gastric adenocarcinoma cases diagnosed in our clinic. The expression characteristics of the cases were evaluated using CerbB2 staining. Simultaneously, CerbB2 expression analyses were performed with the RT-PCR method.
Results: Positive immunoreactivity was observed in 19 of 80 cases (23,75%) in the study conducted using CerbB2 antibody. While 14 of these cases (17.5%) demonstrated weak positive (2+), 5 of them (6.25%) demonstrated strong positive (3+) immunoreactivity. With the RT-PCR method, an increase in gene expression was observed in 12 of 14 weak positive cases (75%). In all 5 strong positive cases, on the other hand, high gene expression was determined. Between CerbB2 immunohistochemical findings and gene expression, 89% compatibility and a statistically significant relationship was determined. While an increase in CerbB2 gene expression, determined by Real Tirme PCR method, was not observed in 57 of 61 cases (96.6%) without CerbB2 immunoreactivity, 4 cases had an increase in CerbB2 expression in comparison to normal.
In our study, a statistically significant relationship was determined between CerbB2 expression and Helicobacter pylori (H. pylori) that is a factor blamed for gastric cancer etiology. The relationship between CerbB2 expression and clinicopathologic prognostic factors has been statistically reviewed; no significant results have been found.
Discussion: Our study has shown that RT-PCR is a method, which might be an alternative to IHC and in ISH methods. It is concluded that a statistically significant relationship might be determined between CerbB2 expression and clinicopathologic prognostic parameters by increasing the case number.

Keywords
Gastric Adenocarcinoma, CerbB2, Immunohistochemistry, Clinicopathologic, PCR

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Introduction

Gastric cancer, although its prevalence and incidence in developed countries has reduced in the last 50 years, still remains the fourth most common cancer worldwide and the second leading cause of death from cancer [1,2]. Although early-diagnosed cases are common in Asian societies; in many societies, patients apply with advanced non-operable or metastatic disease [3]. The development of gastric cancer is a multi-stage, complex process including various genetic and epigenetic changes [4]. Human epidermal growth factor receptor 2 (HER-2/CerbB2) is a member of epidermal growth factor receptor (EGFR) family and codes a transmembrane receptor glycoprotein, which has protooncogene tyrosine kinase activity. When this gene gets phosphorylated, tyrosine kinase activity emerges, signals associated with cell proliferation and differentiation develop. As a result of the overexpression of this receptor protein or the amplification of the gene, many malignancies, particularly breast and gastric cancer emerge [5]. According as the breast cancer, chemotherapy combined with Trastuzumab improves survival in gastric and gastroesophageal junction cancer cases [6].

HER-2 situation must be correctly established in order to include Trastuzumab, developed against HER-2 receptor, in treatment protocols, especially in breast cancer cases [7]. Essential methods applied in routine practice in determining HER-2 in tumor cells are immunohistochemical studies (IHC) and in situ hybridization modalities. While currently the most commonly used in situ hybridization method is fluorescent in situ hybridization (FISH) technic, the results obtained by these modalities are not always totally compatible with each other. Gene amplification does not result in protein expression indicated as immunohistochemical all the time. Gene amplification might not be detected every time in the presence of protein expression [8].

Recently, it has been shown that m-RNA levels in formalin-fixed paraffin-embedded tissues might be quantified using Real-Time Polymerase Chain Reaction (RT-PCR) to determine HER-2 expression [5]. Quantitative RT-PCR is a molecular technic allowing the quantitative assessment of mRNAs transcribed from the gene [9]. RT-PCR is a method providing quantitative results by measuring the fluorescent signal increasing simultaneously with the nucleic acid amplification.

Literature studies on whether the HER-2 situation is a prognostic biomarker report that it is not associated with the clinicopathologic parameters such as age, gender, tumor size, histological differentiation degree; however, it is associated with prognostic parameters such as invasion depth, lymph node involvement, TNM stage [5,10].

Our study aims to validate the immunohistochemistry results based on qualitative data used to determine HER-2 activation in gastric adenocarcinomas with RT-PCR method based on quantitative data, and to evaluate the contribution of this method to confirm the results in grey-zone cases and its relationship with clinicopathologic parameters.

Material and Methods

Case Selection

The cases operated due to gastric tumor and diagnosed in our pathology department as primary gastric adenocarcinoma between 2011 and 2015 were included in our study. Preparations of all cases were re-evaluated by two pathologists and reviewed in regard to histological tumor type, pathologic tumor stage, tumor degree, regional lymph node involvement, histopathologic findings such as the presence of lymphovascular and perineural invasion, concomitant intestinal metaplasia, helicobacter pylori and the staging according to the American Joint Committee on Cancer (AJCC). The size, adequacy of the tissues, and the quality of fixation and processing were evaluated and reviewed. Information on age, gender and clinical data were obtained from the hospital database. Inclusion and exclusion criteria were determined.

The inclusion criteria were as follows:

a. Being diagnosed as gastric adenocarcinoma

b. Being a sporadic case

c. Having multiple paraffin-embedded blocks of tumor tissue after the resection;

The exclusion criteria were as follows:

a. Cases with types such as gastric carcinoma with mucinous/mucinous component, carcinoma with signet ring cell/signet ring cell component, undifferentiated carcinomas, mesenchymal tumors, mixed carcinomas, undifferentiated carcinomas and rare histological variants

b. The presence of cancer in family history

c. Existence of single paraffin block of tumor tissue after the resection

d. Tissues with poor quality of fixation and processing

In consideration of all these examinations, 80 cases with obtainable slides and blocks, diagnosed as gastric carcinoma in Uludağ University Faculty of Medicine, Department of Pathology between 2011-2015 were included in the study group.

The study was approved by the Uludağ University Faculty of Medicine Ethics Board of Clinical Researches dated 19.07.2017 and numbered 2017-10/24.

Immunohistochemical Study

A single block representing the tumor morphology the best was chosen for each case from the formalin-fixed paraffin embedded preparations of 80 cases. Sections 4 micrometer-thick were cut from the blocks for immunohistochemical examination. CerbB2 clone CB11 (1:150, Novocastra, Newcastle, United Kingdom) was used in immunohistochemical examination. After leaving for 1 hour in the oven (ETUV) in immunohistochemical laboratory, blocks were processed in the automatic immunohistochemical staining device (Leica Microsystems, Berlin, Germany). Breast cancer tissue had been used as external control block for CerbB2 antibody.

Immunohistochemical Evaluation

According to the criteria determined in the international and randomly controlled phase 3 study participated involving 122 centers from 24 countries, Trastuzumab for Gastric Cancer (ToGA), CerbB2 antibody staining values were evaluated by scoring between 0 and +3 [3].

Accordingly, resection materials were reviewed as follows:

Score 0: There is no staining or there is membranous staining in less than 10% of the tumor cells

Score 1+: pale/weak; partial membranous staining in more than 10% of the tumor cells

Score 2+: weak/moderate; complete/basolateral membranous

staining in more than 10% of the tumor cells

Score 3+: strong; complete/basolateral/lateral membranous staining in more than 10% of the tumor cells

Score 0-1 was considered as immunoreaction-negative, Score 2+ weak positive immunoreaction and Score 3+ strong positive immunoreaction.

Genetic Analysis

All cases' tissues, which are tumor cell-rich and compatible with normal sites were cut from the blocks in size of 0.2-0.4 cm. Materials were cleared of paraffin by being treated with BIOstic (MO BIO Laboratories, Carlsbad, CA) twice. After resolving paraffin on the tissues with BIOstic, materials were passed through stages of 100%-70% and 40% alcohol and alcohol was vaporized at room temperature. After these steps, RNA isolation from the tumorous tissue samples of 80 patients and 70 normal gastric tissue samples was performed according to the procedure using the commercial kit (Qiagen RNeasy FFPE kit) suitable for the RNA isolation from the paraffin block. The quantity and quality of obtained RNAs were measured using a Spectrometer device (Beckman Coulter). Complementary DNA (cDNA) was obtained from 5 ng of total RNA using ProtoScript M-MuLV First Stand cDNA Synthesis Kit. The reaction mixture was prepared into a 0.2 µl PCR tube for each study specimen. Acquired mixture was mixed by pipetting and span in a centrifuge. The mixture in PCR tube was incubated for 2 hours at 37°C. After the incubation, the obtained cDNAs were transferred in volumes of 90 µl dH2O in each PCR tube containing reaction mixture and mixed, then stored at -20°C until the following step. Primers associated with CerbB2 determined to be used in our study were chosen from the literature. Fluorescent tagged (with Taqman probe) primers were used to determine and evaluate the specific PCR product formed during PCR cycle simultaneously. Ct values of mRNA expressions were obtained from the Abi Step One Plus Real-Time PCR device database. In this study, by determining the Ct value of the Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene used as a housekeeping gene, the data obtained from PCR Array were normalized.

Gene expression results were given as increased/no gene expression

Statistical Analysis

The compatibility of variables to normal distribution was analyzed with the Shapiro- Wilk test. Continuous variables were presented as median (minimum: maximum) values. Categorical variables were reported as n (%). According to test of normality, two independent sample Mann-Whitney test was used in comparison of two groups, independent samples the Kruskal-Wallis test was used in comparisons of three groups, the Pearson chi-square test, Fisher's exact chi-square test and Fisher-Freeman-Halton tests were used in inter-group comparisons of categorical variables. SPSS program (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.) was used for statistical analysis, and p-value of <0.05 was considered as statistically significant.

Results

Demographic and Clinicopathologic Characteristics of Cases

The study included 80 gastric adenocarcinoma cases, 60 males (75%) and 20 females (25%). The age distribution of the cases was between 43 and 88 with the median of 55.2. When histological differentiation degree of tumors (grade) was reviewed, 1 case (1.3%) was well-differentiated (grade 1), 33 were (41.3%) moderate-differentiated (grade 2), 46 (57.5%) were poor differentiated (grade 3). When the cases were evaluated according to pathologic tumor stage (pT), 8 cases were (10%) pT1, 6 (7.5%) were pT2, 50 (62.5%) were pT3, 16 (20%) were pT4. While lymph node involvement was not observed in 22 of the cases (27.5%), 58 (72.5%) had lymph node involvement; 21 cases (26.3%) had lymphovascular invasion and 38 (47.5%) had perineural invasion. Intestinal metaplasia was not observed in 38 cases (47.5%). Among the remaining 42 cases, 35 (43.8%) had complete intestinal metaplasia, 1 (1.3%) had incomplete enteric intestinal metaplasia, 6 (7.5%) had incomplete colonic intestinal metaplasia. H. pylori was detected in 6 cases (7.5%).

Comparison of CerbB2 Expression Level Detected by Immunohistochemistry and RT-PCR Method

According to immunohistochemical examination results, 5 cases (6.2%) had 3+, 14 (17.5%) had 2+, 61 (76.3%) had 0/1+

Table 1. Comparison of cerbB2 staining score with cerbB2 gene expression in cases

CerbB2 immunohistochemistry	CerbB2 gene expression		
	CerbB2 downregulation	CerbB2 upregulation	Total
Negative	57 (%96,6)	4 (3,4)	61
Weak Positive (2+)	2 (%25)	12 (%75)	14
Total	59	16	75

Table 2. Clinicopathological features of the cases according to HER2 PCR groups

	Negative (n=59)	Positive (n=21)	p-value
Pathologic tumor stage			
1	8(%13,60)	0	
2	5(%8,50)	1(%4,80)	0,015[b]
3	31(%52,50)	19(%90,50)	
4	15(%25,40)	1(%4,80)	
Lymph node involvement (positive)	43(%72,90)	15(%71,40)	0,898[d]
H.pylori(positive)	3(%5,10)	3(%14,30)	0,182[e]
Grade			
1	0	1(%4,80)	
2	24(%40,70)	9(%42,90)	0,300[b]
3	35(%59,30)	11(%52,40)	
Diameter			
0	28(%47,50)	8(%38,10)	0,459[d]
1	31(%52,50)	13(%61,90)	
Vascular invasion (positive)	13(%22)	8(%38,10)	0,151[d]
Perineural invasion(positive)	27(%45,80)	11(%52,40)	0 ,602[d]
Intestinal metaplasia			
0	28(%47,50)	10(%47,60)	
1	26(%44,10)	9(%42,90)	0,898[b]
2	1(%1,70)	0	
3	4(%6,80)	2(%9,50)	
Intestinal metaplasia (positive)	31(%52,50)	11(%52,40)	0,990[d]

Data are given as percentages n (%): [b]: Fisher Freeman Halton test [d]: Pearson Chi-Square test [e]: Fisher's Exact test

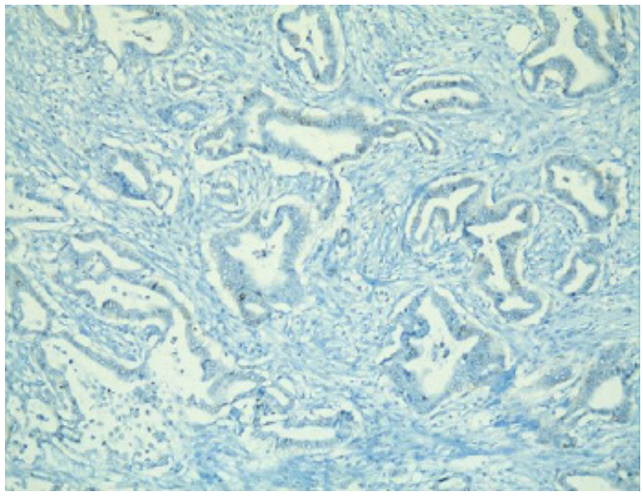

Figure 1. CerbB2 immunohistochemical staining score 0: there is no staining of the tumor cells (IHC, x40)

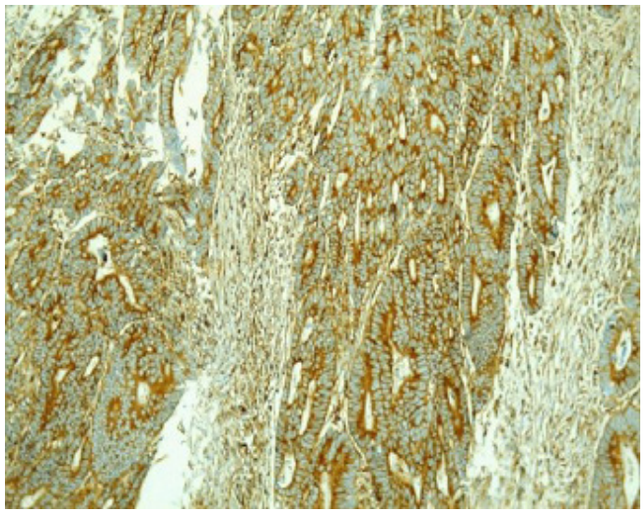

Figure 2. CerbB2 immunohistochemical staining score 2+: moderate, basolateral membranous staining of the tumor cells (IHC, x10)

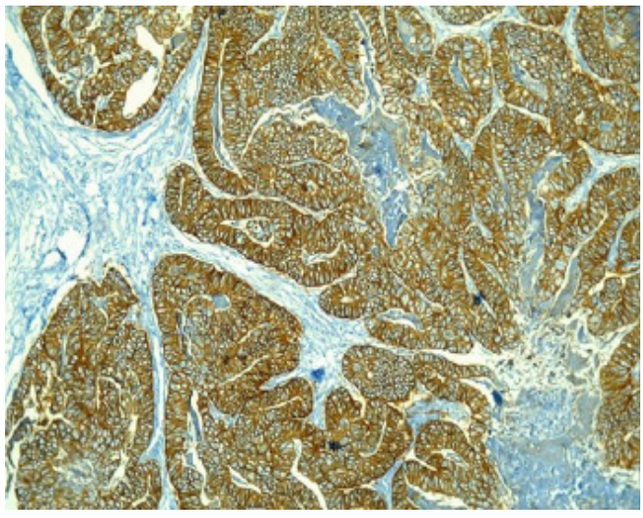

Figure 3. CerbB2 immunohistochemical staining score 3+: strong, complete membranous staining of the tumor cells (IHC, x10)

(Figures 1, 2, 3). While an increase in CerbB2 gene expression determined by the Real Time PCR method was not observed in 57 of 61 cases (96.6%) without CerbB2 immunoreactivity, 4 cases had an increase in CerbB2 expression in comparison to normal. While no difference in gene expression determined by the RT-PCR was observed in 2 of 14 (25%) weak positive cases (staining score 2+), 12 cases (75%) has shown increased gene expression. High CerbB2 gene expression was determined by RT-PCR method in all 5 cases with strong CerbB2 immunoreactivity (staining score 3+). A statistically significant relationship between CerbB2 immunohistochemical findings and gene expression was observed with 89% compatibility ($p<0.05$) (Table 1).

CerbB2 Expression Determined by RT-PCR and Its Relationship with Clinicopathologic Parameters

What is more, when considering the effect of CerbB2 gene expression level determined by RT-PCR on demographic and clinicopathologic parameters in regard to pathologic tumor stage, a statistically significant difference is observed between groups ($p<0.05$). pT3 frequency is observed to be higher in the group without an increase in their CerbB2 gene expression.

No significant relationship has been demonstrated between other demographic and clinicopathologic parameters (age, gender, tumor diameter, tumor degree, regional lymph node involvement, the presence of lymphovascular and perineural invasion, concomitant intestinal metaplasia, helicobacter pylori) and CerbB2 gene expression level (Table 2).

Discussion

Bosard et al. [11] reported that RT-PCR is an alternative method to determine HER-2 situation, by detecting 84% compatibility between HER-2 protein expression and gene amplification in their study, which examines the immunohistochemistry and PCR results of 44 breast cancer cases. Our study aims to contribute to the treatment of gastric adenocarcinoma cases, analyzing HER-2 protein expression at gene amplification level by PCR as an alternative method instead of immunohistochemistry, and revealing the expression in grey zone cases, when immunohistochemistry failed to detect. Zhu et al. [5] has determined CerbB2 positivity with immunohistochemistry as 13.38% (57/426) and with PCR as 11.7% (46/412) in their study on the comparison of quantitative PCR and semi-quantitative immunohistochemistry results. They have reported positive protein expression with PCR method in 4 cases, when immunohistochemistry failed to detect expression and reported 91% compatibility between immunohistochemistry and PCR. In our study, CerbB2 positivity with immunohistochemistry is 23.75% (19/80) and 26% (21/80) with PCR. The compatibility between these two methods, in accordance with the literature, is near 89% ($p<0.05$). Our study determined HER-2 expression with PCR in 4 immunohistochemically negative cases (3.4%). In the literature, it has been reported that HER-2 situation is not associated with clinicopathological parameters such as age, gender, tumor size, histological differentiation degree; however, it is associated with prognostic parameters such as invasion depth, lymph node involvement, tumor stage. Yan et al. [10] determined that HER-2 situation is associated with invasion depth, TNM stage, lymph node involvement

and distant metastasis; however, it is not associated with clinicopathological parameters such as age, gender, tumor size, histological differentiation and localization. Similarly, Zhu et al. [5] showed that HER-2 protein expression is associated with invasion depth, lymph node involvement and TNM stage; nevertheless, it is not associated with tumor localization, age, gender, histological differentiation degree. Rajagopal et al. [12] determined statistically significant relationship between HER-2 expression and male gender in their study (p=0.006). They attributed this result to the greater number of male patients and the higher prevalence of gastric cancer in men. In the same study, a significant relationship has been determined between HER-2 expression and intestinal type and moderately differentiated (Grade 2) tumors. Cases without HER-2 gene expression were determined to have high pT3 frequency in our study (p<0.05). However, no statistical significance was detected in the other comparisons of the groups. No statistically significant relationship has been determined between HER-2 expression and clinicopathological parameters such as age, gender, tumor size, histological differentiation degree, the presence of lymph node involvement, distant metastasis, vascular invasion and perineural invasion. The lack of a statistically significant relationship with parameters such as lymph node involvement, TNM stage, distant metastasis might result from our small study sample. While the study by Zhu et al. [5] is one of the largest-scale (426 cases) studies comparing HER-2 situation with clinicopathological parameters, Yan et al. [10] included 476 patients from several centers.

CerbB2 is the only biomarker used to determine the indication of Trastuzumab, which is one of the standard treatment protocols in advanced stage gastric adenocarcinoma [13,14]. Currently, HER-2 activation is determined using immunohistochemical analysis and in situ hybridization tests in routine practice. Tissues removed after resection and endoscopic intervention are examined primarily with immunohistochemistry. The results are scored in four steps as 0, +1, +2 and +3 according to HER-2 protein quantity stained in tissue. Scores 0 and +1 are defined as HER-2-negative and there is no indication for Trastuzumab treatment in patients with these tumors [15]. While ISH is recommended in cases of immunohistochemical staining score of +2, Trastuzumab treatment is applied to cases with immunohistochemical staining score of +3. However, despite the fact that immunohistochemistry and ISH are used in routine practice, considering their disadvantages such as taking long time, expensiveness and unsuitableness to analyze multiple samples, further studies still continue in an effort to determine a gold standard method [16]. Also, both methods are based on qualitative data and vary in regards to standardization. Therefore, quantitative methods are suitable for the validation of immunohistochemistry and ISH tests.

Conclusion

Consequently, it has been demonstrated that RT-PCR method is an alternative to IHC and ISH methods and these modalities complement each other. Besides, in accordance with the literature, our study has shown that RT-PCR might be a method to clarify the grey zone cases, which may be missed by immunohistochemistry. Undoubtedly, a correct and reliable examination is required due to the direct relationship of HER-2 expression with treatment. In our study, it has been concluded that the relationship between HER-2 expression and clinicopathological parameters might be detected if a larger-scale study is conducted.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

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Conflict of interest

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